

Inhibition of arginine methylation impairs platelet function

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Abstract

Protein arginine methyltransferases (PRMTs) catalyse the transfer of methyl groups to arginine residues in proteins. PRMT inhibitors are novel, promising drugs against cancer that are currently in clinical trials, which include oral administration of the drugs. However, off-target activities of systemically available PRMT inhibitors have not yet been investigated. In this work, we study the relevance of arginine methylation in platelets and investigate the effect of PRMT inhibitors on platelet function and on the expression of relevant platelet receptors. We show that (1) key platelet proteins are modified by arginine methylation, (2) incubation of human platelets with PRMT inhibitors for 4 hours results in impaired capacity of platelets to aggregate in response to thrombin and collagen, with IC_{50} values in the μM range, and (3) treatment with PRMT inhibitors leads to decreased membrane expression and reduced activation of the critical platelet integrin $\alpha_{IIb}\beta_3$. Our contribution opens new avenues for research on arginine methylation in platelets, including the repurposing of arginine methylation inhibitors as novel antiplatelet drugs. We also recommend that current and future clinical trials with PRMT inhibitors consider any adverse effects associated with platelet inhibition of these emerging anti-cancer drugs.

Keywords: antiplatelet, arginine methylation, cancer, inhibitor, platelet, protein arginine methyltransferase.

Platelets, or thrombocytes, are small anucleate discoid cells of 3 μm in diameter and key components of normal haemostasis.¹ In the event of injury, platelets adhere to the injured vessel wall, aggregate with other platelets and combine with soluble fibrinogen and insoluble fibrin to form a platelet plug. Platelets are the second most numerous cells in circulating blood, typically present in $150-440 \times 10^6$ cells/ml and have an average life span of 5-10 days.²

Platelets can be activated by agonists such as thrombin, collagen, adenosine diphosphate (ADP) and von Willebrand factor (vWF). Platelet membranes contain glycoprotein receptors that induce signalling mechanisms inside the cell upon ligand binding to trigger platelet activation. Activated platelets undergo actin cytoskeletal rearrangements to form membrane extensions, called lamellipodia, which facilitate platelet adhesion to vascular

wall structures by increasing the surface area of the platelet.³ Platelets can also adhere to each other through the formation of inter-platelet bridges, in a process known as platelet aggregation.⁴ Platelet aggregation is dependent on the activity of the $\alpha_{IIb}\beta_3$ receptor, the most abundant integrin in human platelets.⁵ Activation of the $\alpha_{IIb}\beta_3$ fibrinogen receptor is the final step in the activation process and the first step towards aggregation, which is amplified by secretion of platelet dense and alpha granules.⁶

Although platelet function is vital to haemostasis, pharmaceutical interventions to decrease the activity of platelets can be very useful in the clinical setting. Patients at risk of cardiovascular disease, including myocardial infarction and stroke, can benefit from antiplatelet therapies that reduce the risk of occlusion of a blood vessel by a thrombus, leading to ischemia. Antiplatelet drugs interfere with mechanisms of platelet activation or aggregation and include aspirin, P2Y₁₂ receptor blockers (such as clopidogrel and ticagrelor) and $\alpha_{IIb}\beta_3$ antagonists.⁷ Antiplatelet drugs have gained visibility in the context of the COVID-19 pandemic,⁸ because overall 17% of COVID-19 patients suffer from venous thromboembolisms.⁹ Although antiplatelet drugs are widely used in the clinics, there is a subtle balancing act between the desirable antiplatelet effects of therapy in patients and a higher risk of bleeding.¹⁰

In a normal physiology setting, the premature activation of platelets is inhibited by endogenous substances secreted by endothelial cells, namely prostacyclin (PGI₂) and nitric oxide (NO). PGI₂ and NO inhibit platelet function through activation of adenylyl cyclase and guanylyl cyclase, respectively, and the corresponding cyclic nucleotide-dependent protein kinase A (PKA) and G (PKG) signalling.¹¹ Vasodilator-stimulated phosphoprotein (VASP) is a key player in platelet signalling pathways through its role as an actin cytoskeleton regulator,¹² and phosphorylation of VASP (by PKA and PKG) is a gold standard of platelet quiescence.¹³

Protein post-translational modifications are key players in signal transduction thanks to their ability to change protein activity, localisation and interactions. Arginine methylation (ArgMe) of proteins consists of the transfer of a methyl group (CH₃) from S-adenosyl-L-methionine onto the side chain guanidino nitrogen of arginine, in an enzymatic reaction catalysed by protein arginine methyltransferases (PRMTs).¹⁴ The addition of a CH₃ group can affect hydrogen bonding interactions of the recipient arginine and produces bulkier and more

hydrophobic methylarginine residues.¹⁵ There are three types of PRMTs, each responsible for a different ArgMe end-product: Type I PRMTs lead to asymmetric dimethylarginine (ADMA); Type II PRMTs produce symmetric dimethylarginine (SDMA); and Type III PRMTs form monomethyl arginine (MMA) only. MMA is produced by all three PRMT types and is often seen as a stable, but intermediate, product in Types I and II PRMT reactions. Type I PRMTs include PRMT1, -2, -3, -4, -6 and -8. PRMT5 and -9 are type II PRMTs. PRMT7 is the only type III PRMT.¹⁶ It is accepted that most of the ArgMe activity in mammalian cells can be attributed to PRMT1.^{17, 18}

PRMTs are key epigenetic regulators or “writers” of epigenetic methylation marks and PRMT inhibitors have been developed over the last two decades to pharmacologically modulate PRMT activity and regulate gene expression.¹⁴ Recently, PRMT inhibitors have entered clinical trials in the oncology setting (identifiers: NCT03666988, NCT02783300, NCT03614728, NCT03573310, NCT03854227 and NCT04089449).¹⁴ Most PRMT inhibitors in clinical trials are administered orally, and therefore systemically.^{19, 20} This raises the obvious question of possible off-target, side effects of ArgMe inhibition in any cell type. There is little documentation available on this issue, but preclinical experiments with rats and dogs revealed moderate changes to haematological profiles, including on the number of platelets, when the animals were treated with the Type I PRMT inhibitor GSK3368715 for up to 28 days.¹⁹ It is therefore timely to investigate any side effects of PRMT inhibitors on platelet function. Furthermore, the extent and role of ArgMe has not been explored in platelets. The aims of the present paper are (1) to reveal the relevance of ArgMe in platelets by describing the platelet “arginine methylome” and (2) to investigate the effect of ArgMe inhibition on platelet function by incubating platelets with PRMT inhibitors.

Results

The platelet arginine methylome reveals key roles in platelet function

To gain an understanding of the scope of protein ArgMe in platelets, we systematically searched available proteomics datasets of platelets for ArgMe. We searched the ProteomeXchange repository for the term “platelet” and identified 13 ProteomeXchange datasets (PXD) suitable for further analysis (Figure 1A and Suppl. Table S1). Using our recently published proteomics workflow,²¹ we identified proteins modified by ArgMe in each of

the 13 projects. We then defined the platelet arginine methylome as those ArgMe sites that were common to three or more projects and we counted 96 sites in 64 proteins that fulfilled this criterium (Suppl. Table S2), including in each of the two components of the $\alpha_{IIb}\beta_3$ receptor.

Analysis of GO terms enriched in this subset of 64 proteins against the platelet proteome background (8527 proteins identified in the 13 PXD projects analysed) revealed enrichment in GO terms associated with platelet activation (Suppl. Table S3). GO terms of platelet aggregation, adhesion, degranulation, exocytosis and secretion, to name a few, were strongly and specifically enriched in the platelet arginine methylome compared to the whole platelet proteome, indicating relevant roles of ArgMe in platelet function. STRING analysis of the arginine methylome mapped out highly interconnected protein networks (Figure 1B). VASP was identified in only 2 PXD projects and was therefore not included in our arginine methylome. Given the central role of VASP in platelet signalling, we nevertheless immunoprecipitated VASP from human platelets and found that, firstly, VASP was recognised by anti-ArgMe antibodies (Suppl. Figure S1) and, secondly, mass spectrometry analysis identified a novel ArgMe site at VASP R10 (Suppl. Figure S1 and Suppl. Table S4).

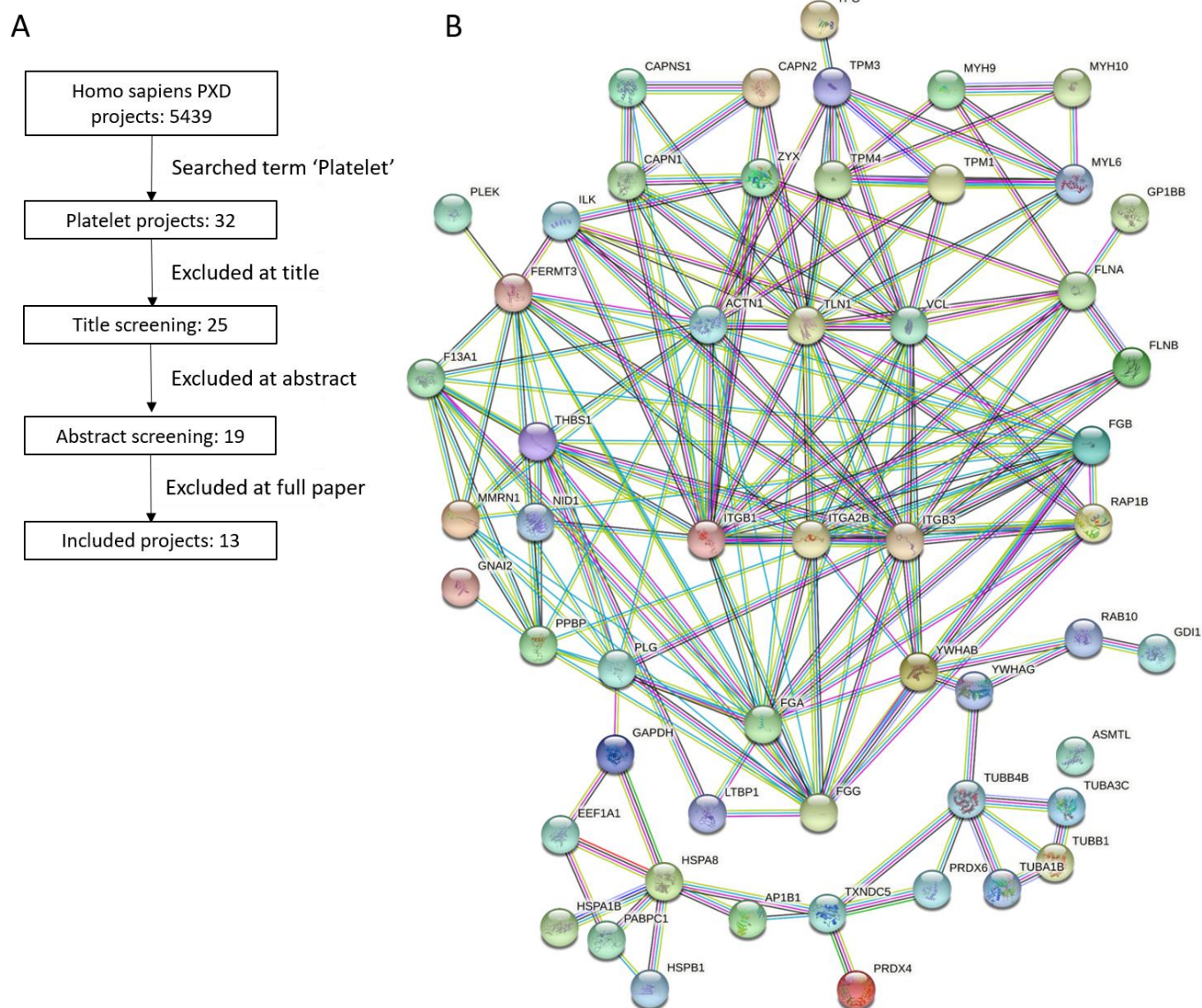


Figure 1. Revealing the platelet arginine methylome. **A.** Decision pipeline towards a systematic analysis of ProteomeXchange datasets of the platelet proteome. **B.** STRING analysis of the platelet arginine methylome showing highly interconnected protein-protein interaction networks. Included in this panel are the proteins that were interconnected (54 out of 64), for a full list see Suppl. Table S2. ITGA2B and ITGB3 in the middle of the panel are the genes coding for the α_{IIb} and β_3 components of the fibrinogen receptor, respectively.

ArgMe can be inhibited in platelets

To provide direct evidence that platelet proteins can be modified by ArgMe, we first tested for expression of the enzyme responsible for S-adenosylmethionine (SAM) synthesis (SAM synthase, MAT2A) and of PRMT1 in platelets. We observed bands at the expected molecular weights (MW), (Figure 2A, left). We also searched for protein ArgMe using western blot and we routinely observed several protein bands recognised by antibodies specific for mono- and di-ArgMe in platelet lysates (Figure 2A, right), although the identity of these proteins remains unknown.

We then incubated platelets with Type I PRMT inhibitors *in vitro* and we observed reduced protein ArgMe profiles after 3-4 hours incubation with μ M concentrations of furamidine and AMI-1 (Figures 2B and 2C and Suppl. Figure S2). For this reason, subsequent functional experiments (see below) were done following incubation of platelets with Type I PRMT inhibitors for 3-4 hours. We observed a dose-response relationship in the inhibition of ArgMe, shown by reduced intensity of ArgMe bands with increasing concentrations of furamidine (Figure 2D). Antibodies #8015 and #8711 were raised against different epitopes and it was therefore not surprising that they seemed to recognise different proteins, this was also shown in the original publication.²² Incubation of platelets with PRMT inhibitors led to increased VASP phosphorylation at S157, a marker of platelet quiescence, although at lower levels than those observed after incubation with established platelet inhibitors such as PGI₂ (Figure 2E).

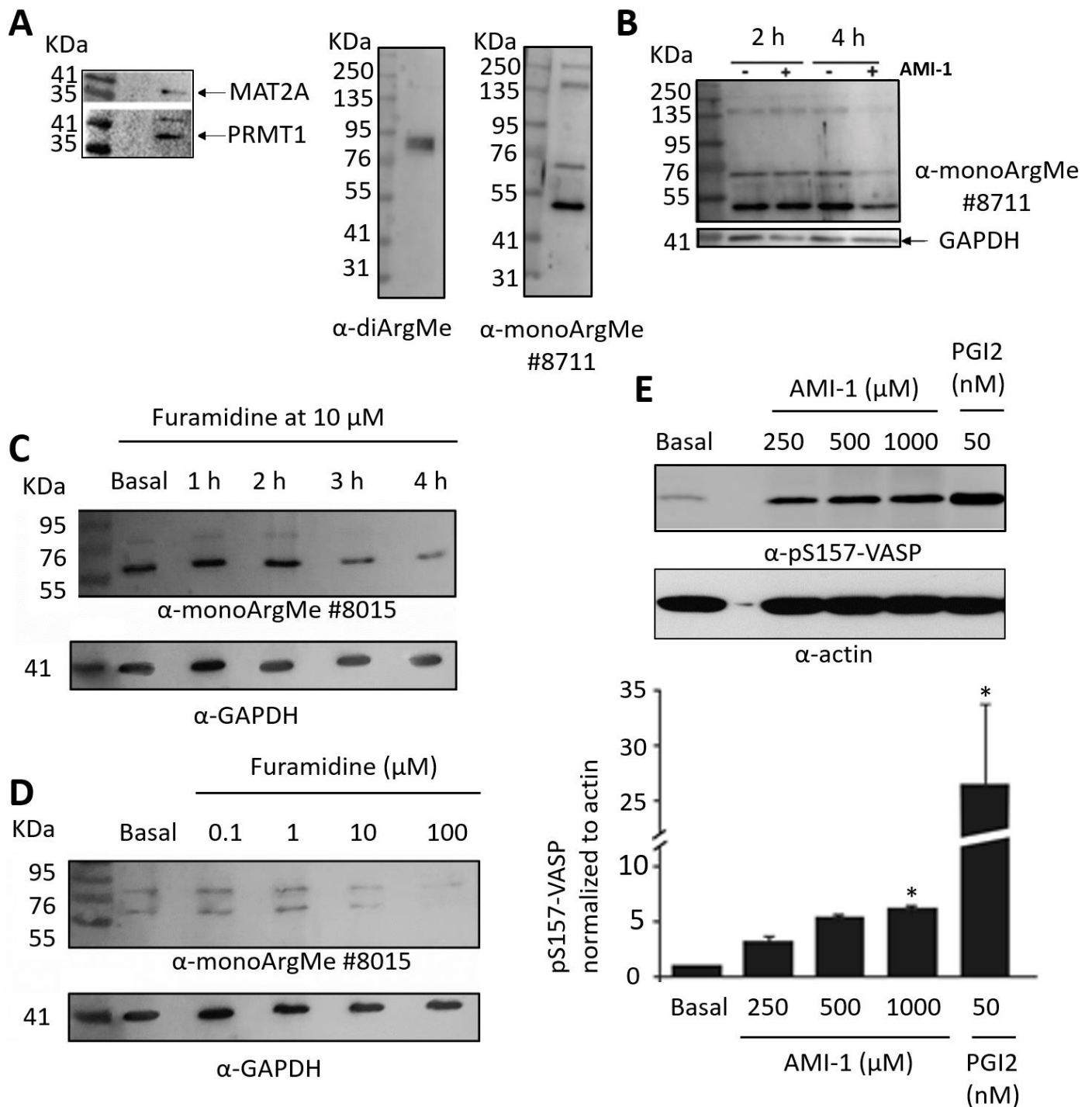


Figure 2. Platelet proteins are modified by ArgMe and incubation of platelets with PRMT inhibitors reduces ArgMe levels. **A.** Detection of MAT2A (expected MW: 43.6 kDa), PRMT1 (expected MW of the main isoform: 42.6 kDa) and ArgMe in platelet lysates using western blot. **B.** Incubation of platelets for 4 hours, but not 2 hours, with 1 mM AMI-1 led to ArgMe inhibition. **C.** Time-course of ArgMe inhibition by 10 μ M furamidine, showing reduced ArgMe after 2-3 hours of incubation. **D.** Dose-response of ArgMe signals with increasing concentrations of furamidine, after a 4-hour incubation

period. **E.** Dose-response of VASP phosphorylation at Ser-157 with increasing concentrations of AMI-1, after a 4-hour incubation period. Representative blot ($n = 3$) and quantification of pS157-VASP relative to actin are shown. Stars indicate statistical significance (* $p < 0.05$).

Inhibition of ArgMe impairs platelet aggregation

Based on the GO term enrichment analysis of our platelet arginine methylome and on our biochemical data, we hypothesised that ArgMe plays a role in platelet aggregation. To test this hypothesis, we performed platelet aggregometry experiments in the presence of increasing amounts of PRMT1 inhibitors. We chose GSK3368715 because it is currently in clinical trials (identifier: NCT03666988).¹⁹ We also used furamidine, AMI-1 and MS023 as Type I PRMT inhibitors that have been developed in preclinical studies and used widely.^{23, 24}

We found dose-dependent inhibition of platelet aggregation after incubation of platelets with Type I PRMT inhibitors *in vitro* for 4 hours. We observed impaired platelet aggregation at low μM furamidine doses in response to thrombin (Figure 3A) or collagen (Suppl. Figure S3). We calculated IC_{50} values for the inhibition of platelet aggregation after thrombin stimulation in the low to mid μM range for furamidine, GSK3368715, MS023 (Figure 3B and 3C) and AMI-1 (Suppl. Figure S4). Because the strongest effects were observed with furamidine, subsequent experiments were performed using this Type I PRMT inhibitor.

Importantly, the inhibition of platelet aggregation by furamidine was at least partly reversible. Indeed, incubation of platelets with a maximum inhibitory dose of furamidine (80 μM) for 2 hours, followed by 2 hours incubation in the absence of furamidine, recovered an average of $33.5 \pm 7.9\%$ platelet aggregation across four donors (Figure 3D, see also Suppl. Figure S5 for representative aggregation curves). The reversibility of furamidine effects strongly suggested that platelets remained active and functional during incubation. To further support this view, we firstly analysed lactate dehydrogenase (LDH) release from platelets incubated with furamidine. LDH analysis showed that platelet integrity was maintained at all concentrations assayed (Suppl. Figure S6). Secondly, we visualised mitochondria in platelets and we found no changes in mitochondrial membrane potential in platelets treated with 80 μM furamidine (Suppl. Figure S7).

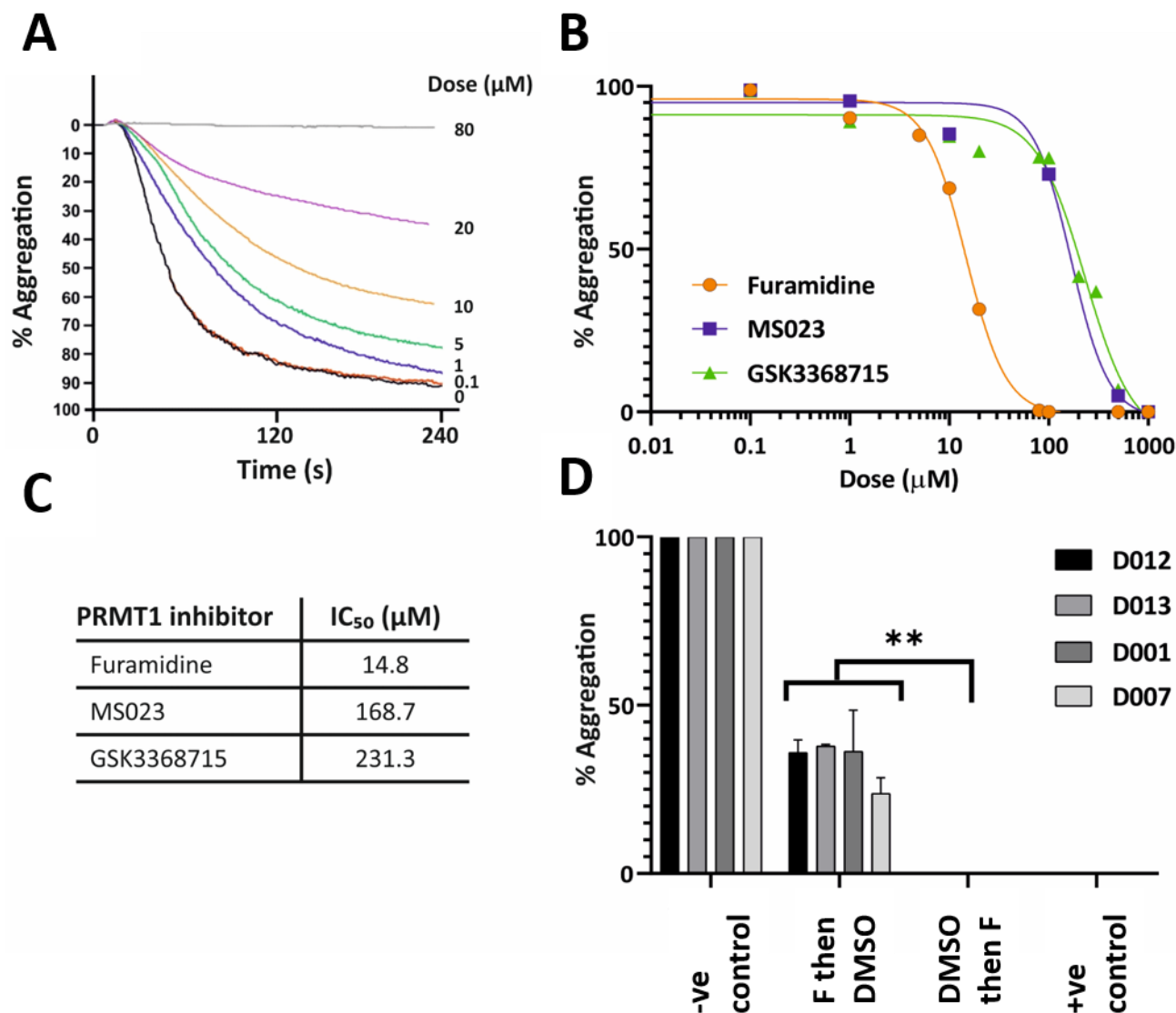


Figure 3. Furamidine causes a dose-dependent inhibition of platelet aggregation. **A.** Effect of furamidine on platelet aggregation stimulated by thrombin (0.1 U/ml). Platelet suspensions were incubated for 4 hours with the indicated concentrations of furamidine prior to stimulation. Traces are average of at least 6 independent experiments. **B** and **C.** Visualisation of and calculated IC₅₀ values for furamidine, MS023 and GSK3368715. **D.** The total inhibition of platelet aggregation by 80 μM furamidine (F) can be partly recovered by removing the inhibitor. Negative (-ve) control is platelets incubated with DMSO for 4 hours at 37°C. Positive (+ve) control is platelets incubated with furamidine (80 μM) for 4 hours. Note the recovery of *ca.* 33.5% aggregation in platelets incubated first with 80 μM furamidine for two hours and

then with DMSO for 2 hours, compared to platelets incubated first with DMSO and then with furamidine. Individual data for four different donors are shown. Stars indicate statistical significance (** $p < 0.005$).

Furamidine treatment leads to altered platelet spreading phenotype

To assess for any effect of furamidine on platelet morphology, we assayed spreading on fibrinogen or collagen coated surfaces. We observed only very small and donor-dependent differences in total platelet numbers or cell surface area in samples treated with furamidine, with a trend towards slightly increased adhesion to fibrinogen and collagen with increased doses of the inhibitor (Figure 4 A-D). Consistent with this, treated platelets spread mainly through stress fibres and lamellipodia (78%) with a minority of actin nodules (16%), compared to a combined phenotype (33% stress fibres and 61% actin nodules) in untreated platelets (Figure 4E).

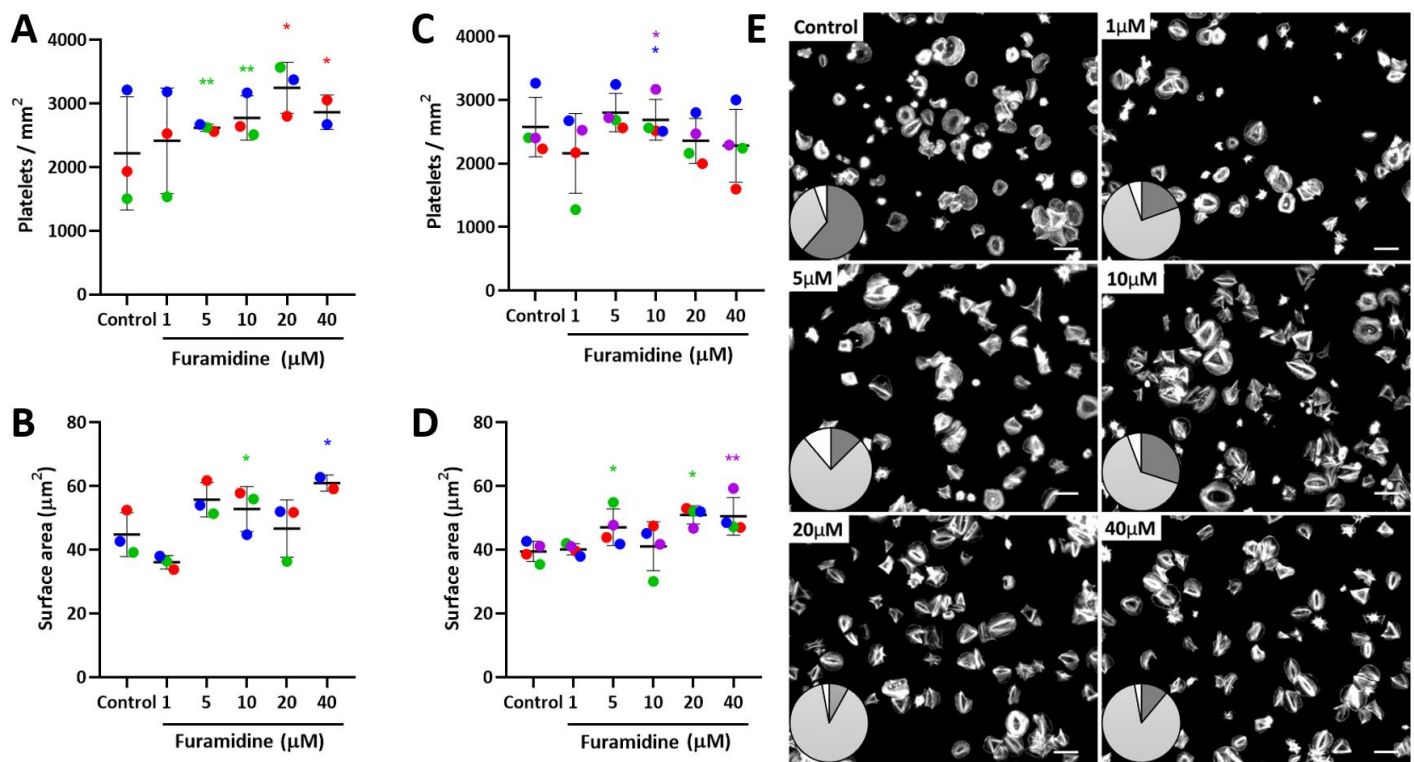


Figure 4. Mild changes in spreading after incubation of platelets with furamidine. A-D. Number of platelets and platelet surface area on spreading onto fibrinogen (A and B) or collagen (C and D) in the absence (control) or in the presence of increasing concentrations of furamidine for 4 hours. Data are shown as the mean \pm SD (error bars), with the data points from each donor ($n = 3$ donors for fibrinogen and $n = 4$ donors for collagen, five slides analysed from each donor) linked

to make donor-specific comparisons. Donors are colour coded. Statistical comparisons are shown between samples from the same donor. Stars indicating statistical relevance (* $p < 0.05$; ** $p < 0.005$) are coloured according to the donor where any difference between treated and control samples was found significant (ANOVA with post hoc Tukey tests). **E.** Representative images of platelets spreading on fibrinogen after treatment with a range of furamidine concentrations (4 hours); all images are from the same donor. Inset: visualisation of spreading phenotype: actin nodules (dark grey), stress fibres (light grey) and mixed phenotype or non-classifiable (white).

Expression and activation of key platelet receptors changes upon treatment with furamidine

To identify any changes in expression or activation of key platelet proteins upon treatment with PRMT inhibitors that could explain the observations above, we performed flow cytometry experiments using platelets incubated with low furamidine concentrations (10 μ M). There were no changes in the expression of the collagen receptors integrin α -2 (CD49b) and glycoprotein VI (GPVI), (Figure 5A). Consistent with the latter, phosphor-tyrosine (pY) signalling was not significantly affected by furamidine (Suppl. Figure S8). However, we observed significant (although small) reductions in the expression of the α -chain of the vWF binding complex GPIb (CD42b) and the critical integrin α _{IIB} (CD41), (Figure 5A). We tested the effect of furamidine on the activation of the α _{IIB} β ₃ receptor by a range of agonists and found that furamidine impaired α _{IIB} β ₃ activation by thrombin, collagen related peptide (CRP), ADP and U46619, an agonist of the thromboxane receptor (Figure 5B).

We also quantified secretion of dense and alpha granules following platelet activation, measured by CD63 and P-selectin, respectively. Both experiments showed a general decrease in antibody binding following furamidine incubation, this decrease was statistically significant when platelets were stimulated with low and high doses of CRP (Figure 5C and 5D). Taken together, our results show that Type I PRMT inhibitors affect expression and activation of critical receptors and impair platelet function.

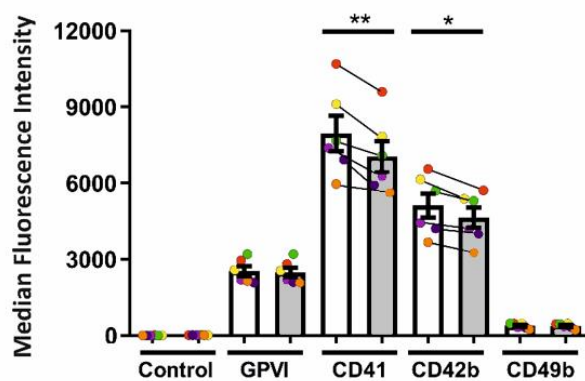
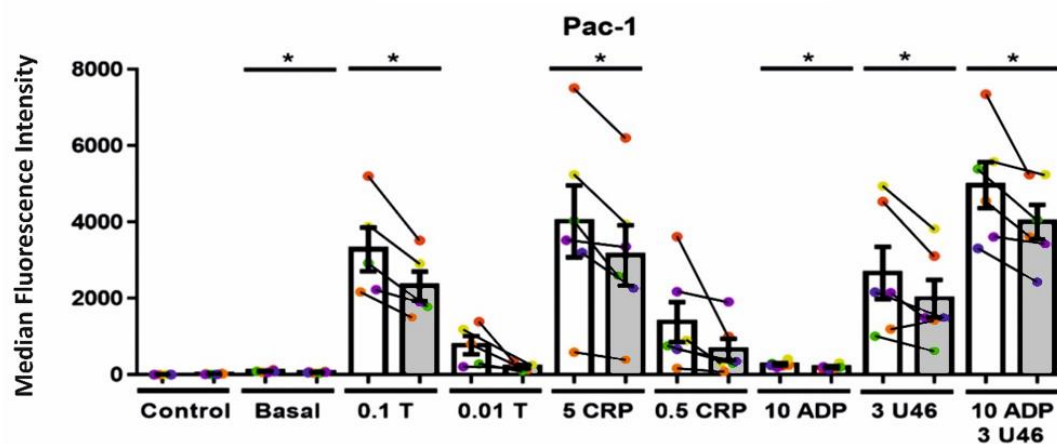
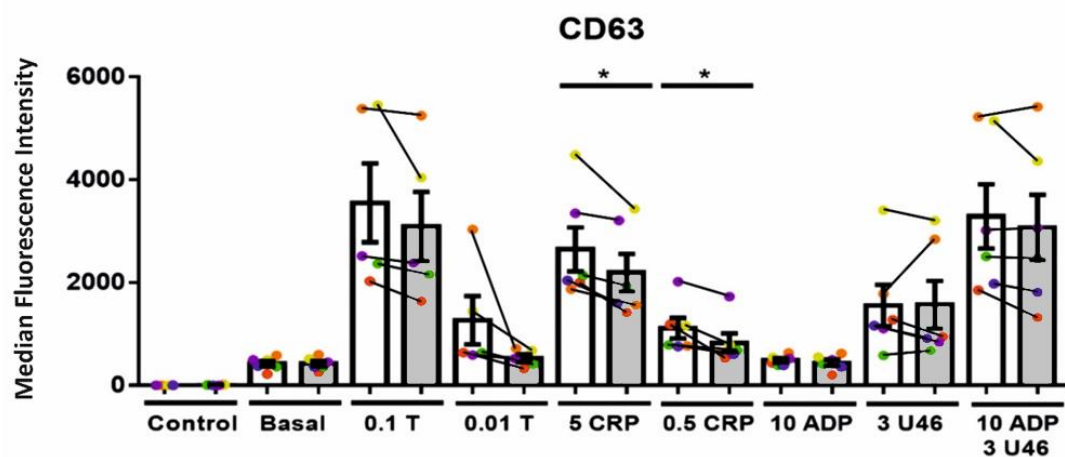
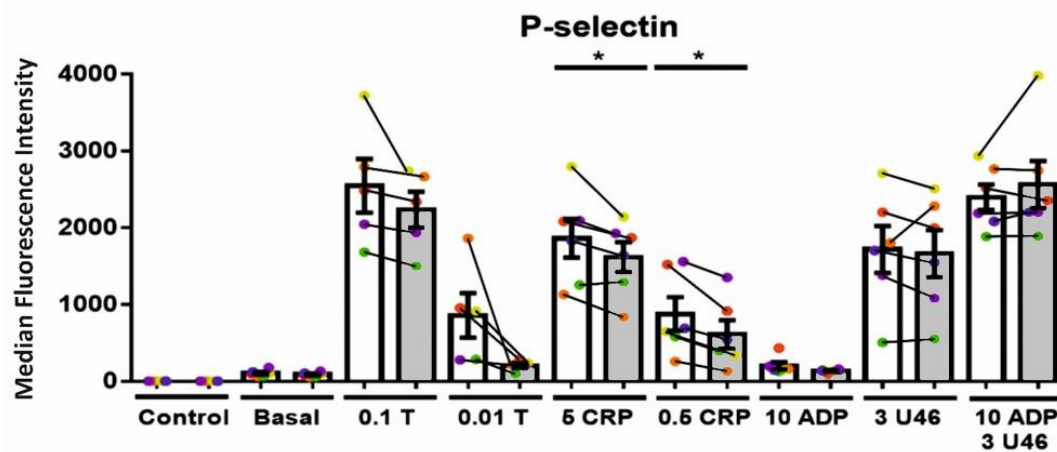
A**B****C****D**

Figure 5. Furamidine treatment leads to changes in receptor expression and granule secretion. Analysis of the effect of 10 μ M furamidine on the expression of (A) key platelet receptors, (B) activated $\alpha_{IIb}\beta_3$ receptor, (C) dense granule secretion and (D) alpha granule secretion. Colours and joined lines denote pairs of control and furamidine-treated platelets and white and grey bars are control and furamidine (at 10 μ M for 4 hours) experiments, respectively. Data are shown as the mean \pm SD (error bars), with the data points from each donor (n = 6 donors) colour-coded and linked to make pairwise comparisons. Stars indicate statistical significance (* p < 0.05; ** p < 0.005, paired Student's t-test).

Discussion

Over the past few years, several groups have begun to identify the set of proteins modified by ArgMe, that is, the arginine methylome, in various cells and tissues,^{22, 25-28} however, the extent and role of ArgMe in platelets has not yet been investigated. Our work indicates that many proteins are modified by ArgMe in platelets. From that standpoint, we decided to investigate the effect of ArgMe inhibition on platelet function. PRMT inhibitors have been hailed as novel, promising drugs for the treatment of several cancer types. This is based on remarkable success in the synthesis of specific inhibitors and on encouraging preclinical and clinical models of disease,^{14, 29-31} which have led to clinical trials by major pharmaceutical companies. The research community has appreciated the need to monitor any side-effects of these emerging class of drugs,³² and our work raises the need to consider platelet inhibition as a possible off-target effect of the systemic administration of PRMT inhibitors in ongoing and subsequent clinical trials.

Incubation of platelets with PRMT inhibitors for 4 hours led to reduced levels of global ArgMe as judged by western analysis. This suggests that there is PRMT activity in washed platelets and that PRMT inhibitors can permeate platelet membranes relatively rapidly. We tested several PRMT1 (or more generally Type I PRMT) inhibitors including AMI-1, MS023, GSK3368715 and furamidine. We expected that MS023 and GSK3368715 would inhibit ArgMe in platelets at the lowest concentrations, given their reported IC₅₀ values against PRMT1 (30 and 3.1 nM, respectively).^{19, 24} However, the largest effect on platelet aggregation was observed in the presence of low μ M concentrations of furamidine, which has a reported PRMT1 IC₅₀ value of 9.4 μ M.²³ We hypothesise that this is due to active transport of furamidine into platelets through the organic cation transporter

1,³³ which is expressed in platelets.³⁴ Platelets cannot be maintained in cell culture and rapidly lose viability *in vitro*, which limits the time that washed platelets can be incubated with PRMT inhibitors. An obvious question that needs to be addressed in the future is the effect of systemic administration of therapeutic doses of PRMT1 inhibitors on platelet function in animal models and clinical trials; especially given previous, limited reports of *in vivo* haematological effects of PRMT inhibitors in the μM range.¹⁹ One would expect that continued oral administration of PRMT inhibitors, for example of GSK3368715, during weeks lead to steady state concentration inside short-lived platelets and consequently ArgMe inhibition. Full-body and tissue specific PRMT knock-outs (KO) have been generated and present a wide range of defects, from embryonic lethality (PRMT1 and -5 KO) to only minor phenotypes,³⁵ however, to our knowledge platelet dysfunction has not been reported in these models. It may be possible that mice platelets are less affected by PRMT downregulation or inhibition and it would be interesting to generate platelet specific PRMT1 KOs in the future to shed light onto this standing matter.

While acknowledging the limitations of working with washed human platelets *in vitro*, we report moderate changes to platelet aggregation and mild changes to spreading and expression of key platelet receptors at low doses of furamidine. We observed impaired platelet aggregation in response to both thrombin and collagen. This is consistent with a decreased expression of α_{IIb} at the cell membrane and reduced activation of the $\alpha_{\text{IIb}}\beta_3$ receptor after incubation of platelets with 10 μM furamidine, both at basal conditions and when platelets were stimulated with agonists (thrombin, CRP, ADP and a thromboxane analogue). Of note, both components of the $\alpha_{\text{IIb}}\beta_3$ receptor were found to be methylated in platelets and it is tempting to speculate that inhibition of ArgMe may jeopardise $\alpha_{\text{IIb}}\beta_3$ docking at the cell membrane. It is known, for example, that ArgMe of certain membrane proteins, such as ion channels, stimulates protein trafficking to the cell membrane.^{36, 37} Alternatively, ArgMe may be associated with receptor stability or heterodimerisation. This can occur for instance through mediating protein-protein interactions as it has been suggested for the endothelial growth factor receptor (EGFR),³⁸ or through cross-talk with neighbouring modifications. For example, Y660 and K669 in β_3 are modified by phosphorylation and ubiquitination, respectively,^{39, 40} and lie in the vicinity of R659, which we have identified as methylated, and there are many examples of cross-talk between ArgMe and neighbouring post-translational modifications that

can affect protein interactions, localisation and stability.¹⁴ In any event and given the central role of the $\alpha_{IIb}\beta_3$ receptor in platelet function, further investigations of $\alpha_{IIb}\beta_3$ ArgMe are warranted. Some ArgMe sites, including those in both components of the $\alpha_{IIb}\beta_3$ receptor, were identified in extracellular domains. While less commonly reported than intracellular ArgMe sites, it is accepted that extracellular receptor domains, for example in EGFR, can be extensively methylated by PRMT1, presumably at the endoplasmic reticulum/Golgi compartments.^{38, 41,}

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We also observed a general trend towards a mild decrease of dense and alpha granule secretion after furamide treatment, as judged by CD63 and P-selectin exposure. This is consistent with the inhibition of platelet aggregation,⁴³ and also with the enrichment in the platelet arginine methylome of GO terms associated with platelet degranulation and secretion. We show very mild effects of furamide on platelet adhesion. We observed a primed platelet phenotype upon treatment with furamide, with a decrease in the number of actin nodules and an increase in the number of stress fibres. This is consistent with the enrichment in GO terms associated with the actomyosin cytoskeleton and stress fibres in the platelet arginine methylome. Proteins with these GO terms in the arginine methylome include myosins, tropomyosins and actinins. We report minor changes in the number of platelets and platelet surface area, but only in a couple of donors and furamide doses. To bring together the significant effects of furamide on platelet aggregation and on receptor expression with the mild spreading phenotype, we reason that furamide must affect signalling downstream of the receptors. There are potentially many pathways that could be affected (for example, cyclic nucleotide dependent signalling, CalDAG-GEFI, Src family tyrosine kinases, cytoskeletal substrates) and decreased receptor levels may not fully explain the observed phenotype. For instance, platelet spreading on fibrinogen could be maintained in the context of reduced $\alpha_{IIb}\beta_3$ activity if ArgMe inhibition enhances GPVI activation by immobilised fibrinogen.⁴⁴ The combination of moderate effects of furamide on platelet aggregation and a mild trend towards increasing platelet adhesion may be of interest towards repurposing PRMT inhibitors as potential, novel antiplatelet agents. However, this idea comes with its own caution note: it is conceivable that PRMT inhibitors also affect the morphology, signalling

and function of other cell entities and these effects cannot currently be predicted, especially for long-term treatments.

Furamide has been used as a specific PRMT1 inhibitor at low μM concentrations.^{23, 45-48} Furamide is also known to have other effects including as an anti-protozoal agent,⁴⁹ DNA binding and inhibitor of tyrosyl-DNA phosphodiesterase 1.⁵⁰ These alternative activities of furamide are unlikely to be relevant in the context of washed or circulating platelets and furamide treatment reduced the intensity of ArgMe bands, which supports the idea that the reported inhibition of platelet function is due to direct inhibition of PRMT activity. Furamide is thought to bind to the substrate pocket on PRMT1 by mimicking the side-chain guanidino group of arginine with involvement of hydrogen bonding and electrostatic interactions,^{23, 51} and thus competes with the substrate and reversibly inhibits PRMT1. Consistent with this, the effects of maximal inhibitory doses of furamide on platelet aggregation were at least partially reversed after a short wash-off.

Taken together, our results open new avenues for research on ArgMe in platelets. Firstly, platelets, being anucleate cells, are a good model for research on ArgMe outside of the nucleus and our data show that tens of proteins, including critical platelet receptors, are modified by ArgMe in human platelets. Secondly, there may be an opportunity to develop inhibitors of specific ArgMe sites of platelet proteins, e.g. $\alpha_{\text{IIb}}\beta_3$, with possible therapeutic applications. Thirdly, we recommend that current and future clinical trials using PRMT inhibitors consider the investigation of platelet function and include the monitoring of possible adverse effects related to platelet inhibition.

Materials and Methods

Ethics Statement

This work was completed in accordance with the University of Hull and Hull York Medical School (HYMS) ethical guidelines. Work with human blood samples, including platelets, was approved by the HYMS ethics committee and was completed under the 'project 1501: The study of platelet activation, signalling and

metabolism'. All participants gave their informed consent prior to their inclusion in the study and the study conformed to the Declaration of Helsinki.

Bioinformatics analysis of ProteomeXchange datasets

The bioinformatic analysis of PXD projects was completed following published protocols.²¹ Briefly, all proteomic raw data in ProteomeXchange as for May 2020 were systematically searched using the following inclusion criteria: 1) inclusion of platelet proteomic data and 2) the data being from human samples. Exclusion criteria were: 1) datasets where enrichment e.g. phosphoenrichment had been performed and 2) unclear labelling of files. Datasets that fulfilled the study criteria were downloaded and mined for protein ArgMe using MaxQuant (v1.6.14.0). ArgMe was set as a variable modification together with Met oxidation and N-terminal protein acetylation. Cys carbamidomethylation was set as a fixed modification. MaxQuant parameters were left as default in searches against the human proteome downloaded from Uniprot (April 2020). The platelet arginine methylome was defined as those ArgMe sites identified in three or more PXD projects, after manual curation of the data to remove likely contaminants (e.g. keratins, immunoglobulins). Enrichment in gene ontology (GO) terms in the platelet arginine methylome was assessed using GOrilla,⁵² using the platelet proteome as background set and the platelet arginine methylome as the target set. STRING analysis of protein networks was done online (<https://string-db.org/>) using high confidence interaction scores (0.7) and all available interaction sources.⁵³

PRMT inhibitors and reagents

MS023 and furamidine were purchased from Tocris, AMI-1 was from Sigma-Aldrich and GSK3368715 was purchased from Cambridge Bioscience. Stock solutions of all ArgMe inhibitors were at 50 mM concentration in DMSO. Thrombin, ADP, fibrinogen and FITC-conjugated phalloidin were from Sigma-Aldrich. Collagen was purchased from Takeda. MitoTracker red CMXRos was from ThermoFisher Scientific. Other reagents were from Sigma-Aldrich unless indicated otherwise.

Platelet isolation and incubation with PRMT inhibitors

Blood (20-80 ml) was collected from healthy donors through venepuncture into acid citrate dextrose (29.9 mM trisodium citrate, 113.8 mM glucose, 72.6 mM NaCl, 2.9 mM citric acid, pH 6.4) and centrifuged at 190×g for

15 min at room temperature to obtain platelet rich plasma, as previously reported.⁵⁴ Platelets were pelleted further by centrifugation at 800×g for 12 min in the presence of 6 mM citric acid, washed (0.036 M citric acid, 0.01 M EDTA, 0.005 M glucose, 0.005 M KCl, 0.09 M NaCl, pH 6.5) and centrifuged as before. Pelleted platelets were resuspended in modified Tyrode's buffer (150 mM NaCl, 5 mM HEPES, 0.55 mM NaH₂PO₄, 7 mM NaHCO₃, 2.7 mM KCl, 0.5 mM MgCl₂, 5.6 mM glucose, pH 7.4) and maintained at 37°C for 30 min in preparation for experiments. Platelets were incubated with PRMT inhibitors or vehicle (DMSO) for 3-4 hours at 37°C unless indicated otherwise.

Platelet aggregation

Platelet aggregation in response to agonists was recorded at a concentration of 3×10⁸ platelets / ml under constant stirring conditions (1000 rpm) for 4 min at 37 °C using Born aggregometry.^{11,55} For the reversibility experiments, we incubated platelets with 80 μM furamidine (or vehicle) for 2 hours, and then on ice for 5 minutes. Platelets were then centrifuged for 50 seconds at 3600×g. The supernatant was removed and the platelets resuspended in modified Tyrode's buffer at room temperature. Only one wash was performed. Platelets were then incubated at 37 °C for the remaining 2 hours with vehicle or 80 μM furamidine as appropriate. Aggregations were completed with 0.1 U/ml thrombin or 10 μg/ml collagen.

Platelet spreading

For platelet spreading analysis, untreated or furamidine-treated platelets (2 × 10⁷/ml) were spread on fibrinogen or collagen (both at 100 μg/ml) coated coverslips at 37°C for the specified times. Platelets were fixed in 4% paraformaldehyde solution for 10 minutes and then permeabilised in 0.1% Triton X-100 for 5 minutes. Slides were stained with FITC-phalloidin for 1 hour for F-actin visualisation, washed, and imaged on a Zeiss Axio Imager fluorescence microscope with a 63× 1.4NA oil immersion objective.^{54, 56} Platelet numbers, actin nodule/stress fibre ratios and surface areas were measured using ImageJ (NIH).

Western blot

Platelet lysates (30-60 μg in 1% SDS) were resolved by 10-12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with antibodies targeting mono-ArgMe (#8015 and #8711, Cell Signalling

Technologies), di-ArgMe (#13522, Cell Signalling Technologies), VASP, α -MAT2A and α -PRMT1 (Abcam) and phosphor-tyrosine (#05-321, Sigma) as appropriate, with GAPDH or β -actin (Thermo) as loading control. Blots were developed using enhanced chemiluminescence reagents (Luminata Forte, Millipore) and signals visualised and captured on a ChemiDoc (Bio-Rad). Blots shown are representative of at least two independent experiments. Quantitative data drawn from western blots are derived from at least $n = 3$ experiments. Densitometry analysis was done using ImageJ (NIH).

Mass spectrometry analysis of VASP

VASP was immunoprecipitated from platelets using specific antibodies (#ab109321, Abcam) and the immunoprecipitate resolved through SDS-PAGE. The putative Coomassie-stained VASP band was digested with Lys-C protease after reduction with dithioerythritol and alkylation with iodoacetamide. The resulting peptides were analysed by LC-MS/MS using an Orbitrap Fusion mass spectrometer with elution from a 50 cm PepMap column over a 35 min gradient. All MS/MS samples were analysed using Mascot (version 2.6.1) and X! Tandem (version CYCLONE 2010.12.01.1). Searches was set up against the UniProt human FASTA database (June 2014, 20259 entries) using Lys-C as the protease. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 3.0 ppm. Cys carbamidomethylation was specified in Mascot and X! Tandem as a fixed modification. Deamidation of Asn and Gln, methylation of Arg, oxidation of Met and acetylation of protein N-termini were specified in Mascot as variable modifications. Glu->pyro-Glu, ammonia-loss, acetylation and Gln->pyro-Glu of the N-termini, deamidation of Asn and Gln, methylation of Arg and oxidation of Met were specified in X! Tandem as variable modifications. Peptide and protein identifications were filtered in Scaffold (version Scaffold_4.8.2) to require a global false discovery rate of <1% at both the protein and peptide level. Protein matches required a minimum of two unique peptide identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm.

Flow cytometry

For flow cytometry analysis of platelet receptors, platelets were incubated with 10 μ M furamidine for a period of 4 hours before fixing (20% paraformaldehyde, BD Phosflow) and staining with antibodies. The antibodies were from BioLegend against the following targets: CD49b (#359309), GPVI (clone HIM3-4, #303302), CD42b (clone HIP1, #303903), CD41 (clone A2A9/6, #359805), active $\alpha_{IIb}\beta_3$ receptor (PAC1, #362803), CD63 (clone H5C6, 353005) and P-selectin (clone AK4, #304905). Analysis of the stained platelets was completed using a LSR FortessaTM from BD Biosciences, and data was analysed with FlowJo.

LDH assays

Lactate dehydrogenase (LDH) assays were done following the instructions of the cytotoxicity detection KitPLUS (LDH) (Roche). Briefly, platelets were lysed in 1% Triton X-100 in 96-well plates (n = 3 independent experiments, 2 technical replicates), the LDH reaction mixture (diaphorase, NAD⁺ and sodium lactate) was added to each well and the plate was incubated at 37°C for 30 minutes in the dark. Reactions were stopped by 1 M HCl and the plate was read at 490 nm with a correction of 680 nm using a spectrophotometer.

Statistical analysis

Data were analysed using paired student's T-tests (flow cytometry experiments), or one-way ANOVA (with post hoc Tukey for pairwise comparisons) as appropriate, with statistical significance defined as $p < 0.05$. Stars in figures define statistical significance.

Supporting Information. Supplementary Figure S1: VASP is modified by ArgMe in human platelets. Supplementary Figure S2. Incubation of platelets with 1 mM AMI-1 for 4 hours leads to inhibition of protein ArgMe. Supplementary Figure S3. Furamidine causes the dose-dependent inhibition of platelet aggregation stimulated by collagen. Supplementary Figure S4. AMI-1 inhibits platelet aggregation. Supplementary Figure S5. Representative example of raw aggregation curves in reversibility experiments. Supplementary Figure S6. LDH assay demonstrates platelet integrity upon incubation with increasing concentrations of furamidine. Supplementary Figure S7. Furamidine does not impair mitochondrial membrane potential. Supplementary Figure S8. Furamidine does not lead to changes in phosphor-tyrosine (pY) signalling. Supplementary Table 1. Identity

of the 13 PXD projects included in our bioinformatics analysis. Supplementary Table 2. Curated list of proteins modified by ArgMe in platelets, that is, the arginine methylome. Supplementary Table 3. GO enrichment analysis in the subset of proteins modified by ArgMe in three or more PXD projects. Supplementary Table 4. Mass spectrometry identification of VASP and R10 methylation.

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Conflict of interest statement: FR and PBA are authors of patent application P031106WO: Arginine methylation inhibitors as novel antiplatelet agents, January 2018.

Abbreviations: adenosine diphosphate (ADP), arginine methylation (ArgMe), lactate dehydrogenase (LDH), prostacyclin (PGI₂), protein arginine methyltransferase (PRMT) von Willebrand factor (vWF)

Author contributions: FR and PBA designed research. NK, BG, FR and PBA critically contributed funding, materials and expertise including software for analysis of platelet spreading (NTK). AJM, DRJR, JSK, AB, FR and PBA performed research. AJM, DRJR, AB, BG, FR and PBA analysed data. AJM, FR and PBA wrote the paper.

References

¹ Kaushansky, K. (2005). The molecular mechanisms that control thrombopoiesis. *Journal of Clinical Investigations*, 115(12), 3339-3347. doi: 10.1172/JCI26674

- ² Li, R., Hoffmeister, K. M. & Falet, H. (2016). Glycans and the platelet life cycle. *Platelets*, 27(6), 505-511. doi: 10.3109/09537104.2016.1171304
- ³ Romaniuk, M.A., Croci, D.O., Lapponi, M.J., Tribulatti, M.V., Negrotto, S., Poirier, F., Campetella, O., Rabinovich, G.A., Schattner, M. (2012) Binding of galectin-1 to $\alpha\text{IIb}\beta_3$ integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis. *FASEB J.* 26(7):2788-98. doi: 10.1096/fj.11-197541.
- ⁴ Ruggeri ZM, Mendolicchio GL. (2007) Adhesion mechanisms in platelet function. *Circ Res.* 100(12):1673-85. doi: 10.1161/01.RES.0000267878.97021.ab.
- ⁵ van den Kerkhof, D.L., van der Meijden, P.E.J., Hackeng, T.M., Dijkgraaf, I. (2021) Exogenous Integrin $\alpha\text{IIb}\beta_3$ Inhibitors Revisited: Past, Present and Future Applications. *Int J Mol Sci.* 22(7):3366. doi: 10.3390/ijms22073366.
- ⁶ Maurice, P., Legrand, C., Fauvel-Lafeve, F. (2004) Platelet adhesion and signaling induced by the octapeptide primary binding sequence (KOGEOGPK) from type III collagen. *FASEB J.* 18(12):1339-47. doi: 10.1096/fj.03-1151com.
- ⁷ Eikelboom, J.W., Hirsh J, Spencer FA, Baglin TP, Weitz JI. (2012) Antiplatelet drugs: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest.* 141(2 Suppl):e89S-e119S. doi: 10.1378/chest.11-2293.
- ⁸ Talasaz, A.H., Sadeghipour, P., Kakavand, H., Aghakouchakzadeh, M., Kordzadeh-Kermani, E., Van Tassell, B.W., Gheymati, A., Ariannejad, H., Hosseini, S.H., Jamalkhani, S., Sholzberg, M., Monreal, M., Jimenez, D., Piazza, G., Parikh, S.A., Kirtane, A.J., Eikelboom, J.W., Connors, J.M., Hunt, B.J., Konstantinides, S.V., Cushman, M., Weitz, J.I., Stone, G.W., Krumholz, H.M., Lip, G.Y.H., Goldhaber, S.Z., Bikdeli, B. (2021) Recent Randomized Trials of Antithrombotic Therapy for Patients With COVID-19: JACC State-of-the-Art Review. *J Am Coll Cardiol.* 77(15):1903-1921. doi: 10.1016/j.jacc.2021.02.035.
- ⁹ Jiménez, D., García-Sánchez, A., Rali, P., Muriel, A., Bikdeli, B., Ruiz-Artacho, P., Le Mao, R., Rodríguez, C., Hunt, B.J., Monreal, M. (2021) Incidence of VTE and Bleeding Among Hospitalized Patients With Coronavirus

- Disease 2019: A Systematic Review and Meta-analysis. *Chest*. 159(3):1182-1196. doi: 10.1016/j.chest.2020.11.005.
- ¹⁰ Vara, D., Tarafdar, A., Celikag, M., Patinha, D., Gulacsy, C.E., Hounslea, E., Warren, Z., Ferreira, B., Koeners, M.P., Caggiano, L., Pula, G. (2020) NADPH oxidase 1 is a novel pharmacological target for the development of an antiplatelet drug without bleeding side effects. *FASEB J*. 34(10):13959-13977. doi: 10.1096/fj.202001086RRR.
- ¹¹ Aburima, A., Berger, M., Spurgeon, B.E.J., Webb, B.A., Wraith, K.S., Febbraio, M., Poole, A.W., Naseem, K.M. (2021) Thrombospondin-1 promotes hemostasis through modulation of cAMP signaling in blood platelets. *Blood*. 137(5):678-689. doi: 10.1182/blood.2020005382.
- ¹² Hubberstey, A.V., Mottillo, E.P. (2002) Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization. *FASEB J*. 16(6):487-99. doi: 10.1096/fj.01-0659rev.
- ¹³ Romero, S., Le Clainche, C., Gautreau, A.M. (2020) Actin polymerization downstream of integrins: signaling pathways and mechanotransduction. *Biochem J*. 477(1):1-21. doi: 10.1042/BCJ20170719.
- ¹⁴ Samuel, S.F., Barry, A., Greenman, J., Beltran-Alvarez, P. (2021) Arginine methylation: the promise of a 'silver bullet' for brain tumours? *Amino Acids*. 53(4):489-506. doi: 10.1007/s00726-020-02937-x.
- ¹⁵ Jain, K., Clarke, S.G. (2019) PRMT7 as a unique member of the protein arginine methyltransferase family: A review. *Arch Biochem Biophys*. 665:36-45. doi: 10.1016/j.abb.2019.02.014.
- ¹⁶ Zurita-Lopez, C.I., Sandberg, T., Kelly, R., Clarke, S.G. (2012) Human protein arginine methyltransferase 7 (PRMT7) is a type III enzyme forming ω -NG-monomethylated arginine residues. *J Biol Chem*. 287(11):7859-70. doi: 10.1074/jbc.M111.336271.
- ¹⁷ Tang, J., Frankel, A., Cook, R.J., Kim, S., Paik, W.K., Williams, K.R., Clarke, S., Herschman, H.R. (2000) PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J Biol Chem*. 275(11):7723-30. doi: 10.1074/jbc.275.11.7723.
- ¹⁸ Wright, T., Wang, Y., Bedford, M.T. (2021) The Role of the PRMT5-SND1 Axis in Hepatocellular Carcinoma. *Epigenomes*. 2021 Mar;5(1):2. doi: 10.3390/epigenomes5010002.

- ¹⁹ Fedoriw, A., Rajapurkar, S.R., O'Brien, S., Gerhart, S.V., Mitchell, L.H., Adams, N.D., Rioux, N., Lingaraj, T., Ribich, S.A., Pappalardi, M.B., Shah, N., Larao, J., Liu, Y., Butticello, M., Carpenter, C.L., Creasy, C., Korenchuk, S., McCabe, M.T., McHugh, C.F., Nagarajan, R., Wagner, C., Zappacosta, F., Annan, R., Concha, N.O., Thomas, R.A., Hart, T.K., Smith, J.J., Copeland, R.A., Moyer, M.P., Campbell, J., Stickland, K., Mills, J., Jacques-O'Hagan, S., Allain, C., Johnston, D., Raimondi, A., Porter Scott, M., Waters, N., Swinger, K., Boriack-Sjodin, A., Riera, T., Shapiro, G., Chesworth, R., Prinjha, R.K., Kruger, R.G., Barbash, O., Mohammad, H.P. (2019) Anti-tumor Activity of the Type I PRMT Inhibitor, GSK3368715, Synergizes with PRMT5 Inhibition through MTAP Loss. *Cancer Cell*. 36(1):100-114.e25. doi: 10.1016/j.ccell.2019.05.014.
- ²⁰ Fong, J.Y., Pignata, L., Goy, P.A., Kawabata, K.C., Lee, S.C., Koh, C.M., Musiani, D., Massignani, E., Kotini, A.G., Penson, A., Wun, C.M., Shen, Y., Schwarz, M., Low, D.H., Rialdi, A., Ki, M., Wollmann, H., Mzoughi, S., Gay, F., Thompson, C., Hart, T., Barbash, O., Luciani, G.M., Szewczyk, M.M., Wouters, B.J., Delwel, R., Papapetrou, E.P., Barsyte-Lovejoy, D., Arrowsmith, C.H., Minden, M.D., Jin, J., Melnick, A., Bonaldi, T., Abdel-Wahab, O., Guccione, E. (2019) Therapeutic Targeting of RNA Splicing Catalysis through Inhibition of Protein Arginine Methylation. *Cancer Cell*. 36(2):194-209.e9. doi: 10.1016/j.ccell.2019.07.003.
- ²¹ Onwuli, D.O., Rigau-Roca, L., Cawthorne, C., Beltran-Alvarez, P. (2017) Mapping arginine methylation in the human body and cardiac disease. *Proteomics Clin Appl*. 11(1-2). doi: 10.1002/prca.201600106.
- ²² Guo, A., Gu, H., Zhou, J., Mulhern, D., Wang, Y., Lee, K.A., Yang, V., Aguiar, M., Kornhauser, J., Jia, X., Ren, J., Beausoleil, S.A., Silva, J.C., Vemulapalli, V., Bedford, M.T., Comb, M.J. (2014) Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Mol Cell Proteomics*. 13(1):372-87. doi: 10.1074/mcp.O113.027870.
- ²³ Yan, L., Yan, C., Qian, K., Su, H., Kofsky-Wofford, S., Lee, W., Zhao, X., Ho, M., Ivanov, I., Zheng, Y.G. (2014) Diamidine compounds for selective inhibition of protein arginine methyltransferase 1. *Journal of Medicinal Chemistry*, 57(6), 2611-2622. doi: 10.1021/jm401884z.
- ²⁴ Eram, M.S., Shen, Y., Szewczyk, M., Wu, H., Senisterra, G., Li, F., Butler, K.V., Kaniskan, H.Ü., Speed, B.A., Dela Seña, C., Dong, A., Zeng, H., Schapira, M., Brown, P.J., Arrowsmith, C.H., Barsyte-Lovejoy, D., Liu, J.,

- Vedadi, M., Jin, J. (2016) A Potent, Selective, and Cell-Active Inhibitor of Human Type I Protein Arginine Methyltransferases. *ACS Chem Biol.* 11(3):772-781. doi: 10.1021/acscchembio.5b00839.
- ²⁵ Geoghegan, V., Guo, A., Trudgian, D., Thomas, B., Acuto, O. (2015) Comprehensive identification of arginine methylation in primary T cells reveals regulatory roles in cell signalling. *Nat Commun.* 6:6758. doi: 10.1038/ncomms7758.
- ²⁶ Larsen, S.C., Sylvestersen, K.B., Mund, A., Lyon, D., Mullari, M., Madsen, M.V., Daniel, J.A., Jensen, L.J., Nielsen, M.L. (2016) Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Sci Signal.* 9(443):rs9. doi: 10.1126/scisignal.aaf7329.
- ²⁷ Musiani, D., Bok, J., Massignani, E., Wu, L., Tabaglio, T., Ippolito, M.R., Cuomo, A., Ozbek, U., Zorgati, H., Ghoshdastider, U., Robinson, R.C., Guccione, E., Bonaldi, T. (2019) Proteomics profiling of arginine methylation defines PRMT5 substrate specificity. *Sci Signal.* 12(575):eaat8388. doi: 10.1126/scisignal.aat8388.
- ²⁸ Lim, Y., Lee, J.Y., Ha, S.J., Yu, S., Shin, J.K., Kim, H.C. (2020) Proteome-wide identification of arginine methylation in colorectal cancer tissues from patients. *Proteome Sci.* 18:6. doi: 10.1186/s12953-020-00162-8.
- ²⁹ Jarrold, J., Davies, C.C. (2019) PRMTs and Arginine Methylation: Cancer's Best-Kept Secret? *Trends Mol Med.* 25(11):993-1009. doi: 10.1016/j.molmed.2019.05.007.
- ³⁰ Bryant, J.P., Heiss, J., Banasavadi-Siddegowda, Y.K. (2021) Arginine Methylation in Brain Tumors: Tumor Biology and Therapeutic Strategies. *Cells.* 10(1):124. doi: 10.3390/cells10010124.
- ³¹ Wu, Q., Schapira, M., Arrowsmith, C.H., Barsyte-Lovejoy, D. (2021) Protein arginine methylation: from enigmatic functions to therapeutic targeting. *Nat Rev Drug Discov.* 20(7):509-530. doi: 10.1038/s41573-021-00159-8.
- ³² Hu, H., Qian, K., Ho, M.C., Zheng, Y.G. (2016) Small Molecule Inhibitors of Protein Arginine Methyltransferases. *Expert Opin Investig Drugs.* 25(3):335-58. doi: 10.1517/13543784.2016.1144747.
- ³³ Ming, X., Ju, W., Wu, H., Tidwell, R.R., Hall, J.E., Thakker, D.R. (2009) Transport of dicationic drugs pentamidine and furamidine by human organic cation transporters. *Drug Metab Dispos.* 37(2):424-30. doi: 10.1124/dmd.108.024083.

- ³⁴ Kim, M.S., Pinto, S.M., Getnet, D., Nirujogi, R.S., Manda, S.S., Chaerkady, R., Madugundu, A.K., Kelkar, D.S., Isserlin, R., Jain, S., Thomas, J.K., Muthusamy, B., Leal-Rojas, P., Kumar, P., Sahasrabudhe, N.A., Balakrishnan, L., Advani, J., George, B., Renuse, S., Selvan, L.D., Patil, A.H., Nanjappa, V., Radhakrishnan, A., Prasad, S., Subbannayya, T., Raju, R., Kumar, M., Sreenivasamurthy, S.K., Marimuthu, A., Sathe, G.J., Chavan, S., Datta, K.K., Subbannayya, Y., Sahu, A., Yelamanchi, S.D., Jayaram, S., Rajagopalan, P., Sharma, J., Murthy, K.R., Syed, N., Goel, R., Khan, A.A., Ahmad, S., Dey, G., Mudgal, K., Chatterjee, A., Huang, T.C., Zhong, J., Wu, X., Shaw, P.G., Freed, D., Zahari, M.S., Mukherjee, K.K., Shankar, S., Mahadevan, A., Lam, H., Mitchell, C.J., Shankar, S.K., Satishchandra, P., Schroeder, J.T., Sirdeshmukh, R., Maitra, A., Leach, S.D., Drake, C.G., Halushka, M.K., Prasad, T.S., Hruban, R.H., Kerr, C.L., Bader, G.D., Iacobuzio-Donahue, C.A., Gowda, H., Pandey, A. (2014) A draft map of the human proteome. *Nature*. 509(7502):575-81. doi: 10.1038/nature13302.
- ³⁵ Blanc, R.S., Richard, S. (2017) Arginine Methylation: The Coming of Age. *Mol Cell*. 65(1):8-24. doi: 10.1016/j.molcel.2016.11.003.
- ³⁶ Baek, J.H., Rubinstein, M., Scheuer, T., Trimmer, J.S. (2014) Reciprocal changes in phosphorylation and methylation of mammalian brain sodium channels in response to seizures. *J Biol Chem*. 289(22):15363-73. doi: 10.1074/jbc.M114.562785.
- ³⁷ Beltran-Alvarez, P., Espejo, A., Schmauder, R., Beltran, C., Mrowka, R., Linke, T., Batlle, M., Pérez-Villa, F., Pérez, G.J., Scornik, F.S., Benndorf, K., Pagans, S., Zimmer, T., Brugada, R. (2013) Protein arginine methyltransferases-3 and -5 increase cell surface expression of cardiac sodium channel. *FEBS Lett*. 587(19):3159-65. doi: 10.1016/j.febslet.2013.07.043.
- ³⁸ Liao, H.W., Hsu, J.M., Xia, W., Wang, H.L., Wang, Y.N., Chang, W.C., Arold, S.T., Chou, C.K., Tsou, P.H., Yamaguchi, H., Fang, Y.F., Lee, H.J, Lee, H.H., Tai, S.K., Yang, M.H., Morelli, M.P., Sen, M., Ladbury, J.E., Chen, C.H., Grandis, J.R., Kopetz, S., Hung, M.C. (2015) PRMT1-mediated methylation of the EGF receptor regulates signaling and cetuximab response. *J Clin Invest*. 125(12):4529-43. doi: 10.1172/JCI82826.
- ³⁹ Hornbeck, P.V., Zhang, B., Murray, B., Kornhauser, J.M., Latham, V., Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res*. 43:D512-20. doi: 10.1093/nar/gku1267.

- ⁴⁰ Akimov, V., Barrio-Hernandez, I., Hansen, S.V.F., Hallenborg, P., Pedersen, A.K., Bekker-Jensen, D.B., Puglia, M., Christensen, S.D.K., Vanselow, J.T., Nielsen, M.M., Kratchmarova, I., Kelstrup, C.D., Olsen, J.V., Blagoev, B. (2018) UbiSite approach for comprehensive mapping of lysine and N-terminal ubiquitination sites. *Nat Struct Mol Biol.* 25(7):631-640. doi: 10.1038/s41594-018-0084-y.
- ⁴¹ Wu, C.C., MacCoss, M.J., Mardones, G., Finnigan, C., Mogelsvang, S., Yates, J.R. 3rd, Howell, K.E. (2004) Organellar proteomics reveals Golgi arginine dimethylation. *Mol Biol Cell.* 15(6):2907-19. doi: 10.1091/mbc.e04-02-0101.
- ⁴² Epstein, D.M., Buck, E. (2015) Old dog, new tricks: extracellular domain arginine methylation regulates EGFR function. *J Clin Invest.* 125(12):4320-2. doi: 10.1172/JCI85001.
- ⁴³ Golebiewska, E.M., Poole, A.W. (2015) Platelet secretion: From haemostasis to wound healing and beyond. *Blood Rev.* 29(3):153-62. doi: 10.1016/j.blre.2014.10.003.
- ⁴⁴ Mangin, P.H., Onselaer, M.B., Receveur, N., Le Lay, N., Hardy, A.T., Wilson, C., Sanchez, X., Loyau, S., Dupuis, A., Babar, A.K., Miller, J.L., Philippou, H., Hughes, C.E., Herr, A.B., Ariëns, R.A., Mezzano, D., Jandrot-Perrus, M., Gachet, C., Watson, S.P. (2018) Immobilized fibrinogen activates human platelets through glycoprotein VI. *Haematologica.* 103(5):898-907. doi: 10.3324/haematol.2017.182972.
- ⁴⁵ Iderzorig, T., Kellen, J., Osude, C., Singh, S., Woodman, J.A., Garcia, C., Puri, N. (2018) Comparison of EMT mediated tyrosine kinase inhibitor resistance in NSCLC. *Biochem Biophys Res Commun.* 496(2):770-777. doi: 10.1016/j.bbrc.2018.01.069.
- ⁴⁶ Jin, S., Mi, Y., Song, J., Zhang, P., Liu, Y. (2018) PRMT1-RBM15 axis regulates megakaryocytic differentiation of human umbilical cord blood CD34+ cells. *Exp Ther Med.* 15(3):2563-2568. doi: 10.3892/etm.2018.5693.
- ⁴⁷ Samuel, S.F., Marsden, A.J., Deepak, S., Rivero, F., Greenman, J., Beltran-Alvarez, P. (2018) Inhibiting Arginine Methylation as a Tool to Investigate Cross-Talk with Methylation and Acetylation Post-Translational Modifications in a Glioblastoma Cell Line. *Proteomes.* 6(4):44. doi: 10.3390/proteomes6040044.

- ⁴⁸ Wang, Y., Li, X., Ge, J., Liu, M., Pang, X., Liu, J., Luo, C., Xu, Y., Zhao, Q. (2021) The methyltransferase PRMT1 regulates γ -globin translation. *J Biol Chem.* 296:100417. doi: 10.1016/j.jbc.2021.100417.
- ⁴⁹ Patrick, D.A., Bakunov, S.A., Bakunova, S.M., Wenzler, T., Brun, R., Tidwell, R.R. (2014) Antiprotozoal activity of dicationic 3,5-diphenylisoxazoles, their prodrugs and aza-analogues. *Bioorg Med Chem.* 22(1):559-76. doi: 10.1016/j.bmc.2013.10.050.
- ⁵⁰ Antony, S., Marchand, C., Stephen, A.G., Thibaut, L., Agama, K.K., Fisher, R.J., Pommier, Y. (2007) Novel high-throughput electrochemiluminescent assay for identification of human tyrosyl-DNA phosphodiesterase (Tdp1) inhibitors and characterization of furamidine (NSC 305831) as an inhibitor of Tdp1. *Nucleic Acids Res.* 35(13):4474-84. doi: 10.1093/nar/gkm463.
- ⁵¹ Zhang, J., Qian, K., Yan, C., He, M., Jassim, B.A., Ivanov, I., Zheng, Y.G. (2017) Discovery of Decamidine as a New and Potent PRMT1 Inhibitor. *Medchemcomm.* 8(2):440-444. doi: 10.1039/C6MD00573J.
- ⁵² Eden, E., Navon, R., Steinfeld, I., Lipson, D., Yakhini, Z. (2009) GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics.* 10:48. doi: 10.1186/1471-2105-10-48.
- ⁵³ Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K.P., Kuhn, M., Bork, P., Jensen, L.J., von Mering, C. (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43:D447-52. doi: 10.1093/nar/gku1003.
- ⁵⁴ Riley, D.R.J., Khalil, J.S., Naseem, K.M., Rivero, F. (2020) Biochemical and immunocytochemical characterization of coronins in platelets. *Platelets.* 31(7):913-924. doi: 10.1080/09537104.2019.1696457.
- ⁵⁵ Riley, D.R.J., Khalil, J.S., Pieters, J., Naseem, K.M., Rivero, F. (2020) Coronin 1 Is Required for Integrin β 2 Translocation in Platelets. *Int J Mol Sci.* 21(1):356. doi: 10.3390/ijms21010356.
- ⁵⁶ Atkinson, L., Yusuf, M.Z., Aburima, A., Ahmed, Y., Thomas, S.G., Naseem, K.M., Calaminus, S.D.J. (2018) Reversal of stress fibre formation by Nitric Oxide mediated RhoA inhibition leads to reduction in the height of preformed thrombi. *Sci Rep.* 8(1):3032. doi: 10.1038/s41598-018-21167-6.

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Inhibition of arginine methylation impairs platelet function

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Brief synopsis:

Protein arginine methyltransferases (PRMT) inhibitors are very novel drugs against cancer that are currently in clinical trials, however, off-target activities of systemically available PRMT inhibitors have not yet been fully investigated. In this work, we have studied the effect of PRMT inhibitors on human platelet molecular and functional phenotypes. We recommend that current and future clinical trials with PRMT inhibitors consider any effects associated with platelet inhibition of these promising anti-cancer drugs.

